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The pathological effect of *Helicobacter pylori* infection on liver tissues in mice

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Abstract

Helicobacter pylori infection is associated with chronic gastritis, peptic ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma. Some reports also suggest that it causes extragastric disease, including hepatitis. In this study, the pathological changes in the liver and gall bladder in *H. pylori*-colonized C57BL/6 mice were investigated. Twenty mice were inoculated orally with *H. pylori* strain SS1, and ten controls were injected with phosphate-buffered saline. Gastric colonization with *H. pylori* was assessed at 2 months after inoculation. Mice were examined at 8 months by histopathology, culture for *H. pylori*, and PCR for specific *H. pylori* genes. All C57BL/6 mice infected with *H. pylori* for 8 months developed severe gastric mucosal inflammation. Three mice showed mild-to-moderate mutifocal hepatitis. The gall bladder mucosa of one *H. pylori*-infected mouse showed thickening of the mucous membrane with mild submucosal lymphocytic infiltration. *H. pylori* was observed morphologically in four liver specimens and six gall bladders from infected mice by immunohistochemistry. Specific *H. pylori* genes were also detected in six liver samples from infected mice, six samples of bile, and two blood samples by nested PCR. Thus, *H. pylori* inoculated orally may reach the hepatobiliary system and cause inflammation as an independent aetiological factor. The pathway to the liver may be via the blood or the biliary system.

Keywords: C57BL/6 mouse, *Helicobacter pylori*, hepatitis, hepatocellular, nested PCR
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Introduction

During the past few years, infections due to *Helicobacter* spp. have been reported to be associated with liver disease in some animal species. Examples are *Helicobacter canis* in dogs, *Helicobacter pullorum* in poultry, and *Helicobacter hepaticus* and *Helicobacter bilis* in mice. It is of note that *H. hepaticus* infection has been found to increase markedly the risk of hepatic carcinoma, in particular in inbred strains of mice [1]. This type of murine model has proved useful in elucidating the molecular mechanism of hepatic cancer associated with *Helicobacter* infection [2]. The human gastric pathogen *Helicobacter pylori*, which causes a persistent infection, is considered to be a type I carcinogen because of its role in the development of gastric carcinoma and gastric mucosa-associated lymphoid tissue lymphoma [3]. It has been suggested that in humans, as in animals, *Helicobacter* spp. may also colonize the liver and induce chronic hepatic diseases, especially hepatocellular carcinoma (HCC).

Recent studies have shown that cells or fragments of *Helicobacter* spp. may be found in the livers of patients with primary sclerosing cholangitis and also those with primary biliary cirrhosis [4,5]. Several separate research groups have reported that DNA of *Helicobacter* spp. was present in liver tissue from patients with primary HCC [6–8]. DNA sequencing showed that the *Helicobacter* spp. involved were closely related to *H. pylori*. De Magalhães Queiroz *et al.* [9] reported the culture of *H. pylori* from one patient with Wilson's disease with cirrhosis and cholestasis. These reports suggested that *H. pylori* may play an important role in the development of hepatobiliary diseases in humans. In our previous study, we found that 60% (nine of 15) of the liver specimens from patients with HCC were positive for the 16S rRNA gene of *Helicobacter* spp. Sequence analysis and comparison showed that these *Helicobacter* spp. were closely related to *H. pylori*. Immunohistochemical staining also showed evidence of *H. pylori* [10,11].

Although *H. pylori* has been considered to be associated with hepatobiliary diseases in humans, there are no detailed

studies of the pathological changes in the hepatobiliary system in *H. pylori*-infected animal models. In the present study, we examined the changes in the liver and gall bladder in *H. pylori*-colonized C57BL/6 mice.

Materials and Methods

Bacterial strains

H. pylori strain SS1 (a cagA-positive, vacA s2-m2 strain) was grown on GAB-Camp agar consisting of 10% (v/v) sterile sheep blood and Skirrow's supplement (10 μg/mL vancomycin, 5 μg/mL trimethoprim lactate, 2500 IU/L polymyxin B, and 5 μg/mL amphotericin B). Plates were incubated for 48 h at 37°C under micro-aerophilic conditions [12]. The bacteria were harvested in phosphate-buffered saline (PBS), visualized by Gram stain and phase microscopy for purity, morphology, and motility, and tested biochemically for urease, catalase and oxidase acitivity. The cells were centrifuged at 3000 ***g*** for 10 min, and resuspended in PBS at a final concentration of 1×10^9 CFU/mL.

Animals

Conventional C57BL/6 male mice, 6–8 weeks old, were housed in micro-isolator caging, and fed with pelleted rodent chow and water *ad libitum* for the duration of the experiment. Twenty C57BL/6 mice were inoculated orally three times at 2-day intervals with 0.5 mL of PBS containing 5×10^8 CFU of *H. pylori*. Ten age-matched and sex-matched C57BL/6 mice were used as control animals, and were inoculated orally with PBS alone. Gastric colonization with *H. pylori* was assessed at 2 months and 8 months post-inoculation. Necropsy for histopathology and bacterial isolation was performed at 8 months.

Bacterial isolation

Mucosal scrapings of gastric antrum and body were obtained aseptically at necropsy, and were rinsed in PBS, smeared directly on GAB-Camp agar, and incubated at 37°C under micro-aerophilic conditions. Samples of blood, bile and liver were also collected aseptically at necropsy and cultured. Blood obtained by cardiac puncture and bile were inoculated directly onto blood agar plates. The liver tissues were ground in sterile tissue grinders and were applied directly to GAB-Camp agar plates. Plates were maintained for 10 days before a result of no growth was recorded. The presence of *H. pylori* on the culture plates was confirmed by colony morphology, urease, catalase and oxidase reactions, Gram staining, and PCR.

Histopathology and immunohistochemistry

Murine stomach, gall bladder and liver tissues were fixed in neutral buffered 10% formalin, embedded in paraffin, sectioned at 4 μm, and stained with haematoxylin and eosin. Colonization by *H. pylori* was also confirmed by immunostaining with anti-*H. pylori* antibody (DAKO) and carbol fuchsin staining [13].

DNA extraction

DNA was extracted from gastric mucosa, liver tissue and gall bladder using a fast method. Tissue (50 mg) was homogenized and centrifuged at 8000 ***g*** for 10 min. The DNA was extracted as previously described [10].

To extract bacterial DNA from serum and bile, 250 μL of fluid was mixed with 250 μL of extraction buffer [22], and incubated at 37°C for 2 h; routine phenol–chloroform extraction and ethanol precipitation were then performed.

PCR amplification

Samples from hepatic tissue, blood and bile were amplified by nested PCR for the *H. pylori*-specific 16S rRNA gene. The two pairs of primers amplified a product of approximately 1020 bp. The other six pairs of specific primers were used to look for the 26-kDa, *vacA* and *cagA* genes of *H. pylori*. Table 1 shows the nucleotide sequences of the primers used, the PCR conditions, and the size of the amplified fragments. Samples from the gastric mucosa were amplified only by outer primers of the above four genes. The amplified products were analysed with 1.5% (w/v) agarose gel, which was stained with ethidium bromide and observed under short-wavelength ultraviolet light.

Sequencing and sequence analysis

The amplicons of the *Helicobacter* 16S rRNA gene were purified using an agarose gel DNA extraction kit (Roche). Sequence analysis was performed with an Applied Biosystems DNA Sequencer. Sequence comparison was carried out using the BLAST program and the GenBank databases.

Statistical analyses

To compare the occurrence of *Helicobacter* genes in the two groups, the chi-square test was used. Significance was assigned to values of $p < 0.05$.

Results

Clinical and gross findings

Two mice died at week 2 after infection. The experiment was continued with the remaining 18 mice. All mice inoculated orally with *H. pylori* became slightly to moderately debilitated and emaciated at month 8. In contrast, the mice inoculated with PBS did not show any clinical signs, or gross lesions at necropsy at month 8.

TABLE 1. Oligonucleotide primers used to amplify the 16S rRNA and *Helicobacter pylori*-specific genes

Gene	Sequence (5′–3′)	Cycle	Amplicon
16S rRNA			
First pair of primers	GCTATGACGGGTATCC ACTTCACCCCAGTCGCTG	94°C for 1 min, 50°C for 1 min, 72°C for 1 min, 35 cycles	1200 bp
Nested primers	GACACGGTCCAGACTCCTA TGGCTGATTTGCGATTA	94°C for 1 min, 52°C for 1.5 min, 72°C for 2 min, 30 cycles	1020 bp
26 kDa			
First pair of primers	TGGCGTGTCTATTGACAGCGAGC CCTGCTGGGCATACTTCACCATG	94°C for 30 s, 68°C for 30 s, 72°C for 1 min, 35 cycles	298 bp
Nested primers	GTCTTTCCCTATGGTGGC GCATTTCATCTGCATTCCT	94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 30 cycles	167 bp
cagA			
First pair of primers	ACGATTGGAACGCCACC CGCCATTTGTAACGCCTA	94°C for 1 min, 52°C for 1 min, 72°C for 1 min, 35 cycles	588 bp
Nested primers	ATAATGCTAAATTAGACAACTTGAGCGA TTAGAATAATCAACAAACATCACGCCAT	94°C for 1 min, 52°C for 1 min, 72°C for 1 min, 30 cycles	297 bp
vacA			
First pair of primers	GCCCCAGGAAACATTG ACACCAGCCTTAAACTCAA	94°C for 1 min, 50°C for 1 min, 72°C for 1 min, 35 cycles	617 bp
Nested primers	GCCCCAGGAAACATTG CATAACTAGCGCCTTGCAC	94°C for 1 min, 52°C for 1 min, 72°C for 1 min, 30 cycles	352 bp

Recovery of *H. pylori* by culture

For assessment of colonization by *H. pylori*, three inoculated mice underwent necropsy at month 2, and gastric tissues were obtained for *H. pylori* culture. *H. pylori* was isolated from these three mice, thus demonstrating successful colonization. At month 8 after infection, *H. pylori* was isolated from the stomachs of eight of the remaining 15 mice, but was not recovered from blood, liver, or gall bladder (Table 2). In control mice inoculated with PBS, *H. pylori* was never recovered from stomach, blood, or liver ($p < 0.01$).

Histopathology

All 15 C57BL/6 mice infected with *H. pylori* for 8 months developed moderate to severe gastric mucosal inflammation. Two mice developed macroscopic ulceration of the gastric mucosa. Six of the 15 mice showed gastric atrophy with mucosal cell proliferation and squamous transformation (Fig. 1). No lymphoma was observed in these areas.

Three mice inoculated with *H. pylori* showed mild to moderate mutifocal hepatitis in parenchymal, perivenular or periportal areas. The lesions consisted of multifocal or coalescing coagulative necrosis of hepatocytes with inflammatory cell infiltration at the periphery. The necrotic foci involved a few or many hepatocytes, and the inflammatory cells were mainly lymphocytes and neutrophils. The punctate lesions were often adjacent or in close proximity to central veins and/or intralobular venules. Ballooning degeneration

TABLE 2. Liver and gastric pathology and *Helicobacter pylori* culture, immunohistochemical staining for *H. pylori* and *Helicobacter* PCR results in stomach, blood, bile and liver samples

			***H. pylori* culture**				***H. pylori* staining**			***Helicobacter* PCR results**			
Mouse	**Gastric pathology**	**Liver pathology**	**Stomach**	**Blood**	**Bile**	**Liver**	**Stomach**	**Gall bladder**	**Liver**	**Stomach**	**Blood**	**Bile**	**Liver**
1	Gastritis	Normal	+	–	–	–	+	–	–	+	–	–	–
2	Gastritis and gastric atrophy	Normal	–	–	–	–	+	+	–	+	–	+	+
3	Gastritis and ulceration	Mutifocal hepatitis, ballooning degeneration	+	–	–	–	+	+	+	+	+	+	+
4	Gastritis	Normal	+	–	–	–	+	–	–	+	–	–	–
5	Gastritis	Normal	–	–	–	–	–	–	–	–	–	–	–
6	Gastritis	Normal	+	–	–	–	+	–	–	+	–	–	–
7	Gastritis and gastric atrophy	Normal	–	–	–	–	+	+	+	+	–	+	+
8	Gastritis and gastric atrophy	Mutifocal hepatitis, ballooning degeneration	+	–	–	–	+	+	+	+	–	+	+
9	Gastritis	Normal	–	–	–	–	+	–	–	+	–		–
10	Gastritis and ulceration	Normal	+	–	–	–	+	+	–	+	+	+	+
11	Gastritis and gastric atrophy	Mutifocal hepatitis	+	–	–	–	+	+	+	+	–	+	+
12	Gastritis	Normal	+	–	–	–	+	–	–	+	–	–	–
13	Gastritis and gastric atrophy	Normal	–	–	–	–	–	–	–	+	–	–	–
14	Gastritis and gastric atrophy	Normal	–	–	–	–	+	–	–	+	–	–	–
15	Gastritis	Normal	–	–	–	–	+	–	–	+	–	–	–

According to the results of *H. pylori* culturing, staining, and PCR, 14 of 15 inoculated mice showed colonization by *H. pylori* of the gastric mucosa. *H. pylori* DNA was detected in six liver samples from these 14 infected mice. The gall bladder and bile from the above six mice were also positive for *H. pylori* staining and gene amplification. Two blood samples from these mice were positive for *H. pylori* genes. These results showed good correlation between the detection methods.

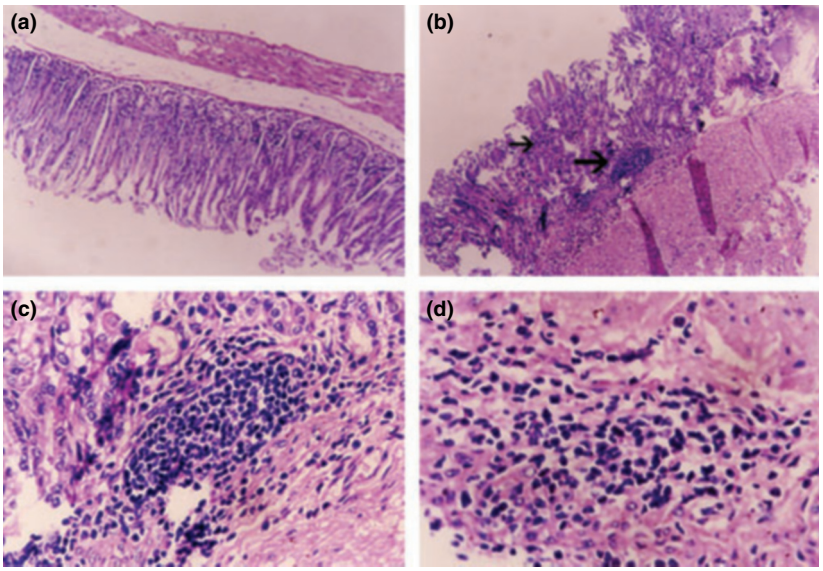

FIG. 1. Gastric inflammation associated with *Helicobacter pylori* infection (haematoxylin and eosin staining). (a) Normal gastric musoca of uninfected animal. (b–d) Significant widespread lymphoplasmacytic gastritis of the entire mucosa and parts of the submucosa in the SS1-infected C57BL/6 mice. Original magnifications: ×300 (a, b) and ×600 (c, d). Arrows indicate infiltrating inflammatory cells in the mucosa and submucosal regions.

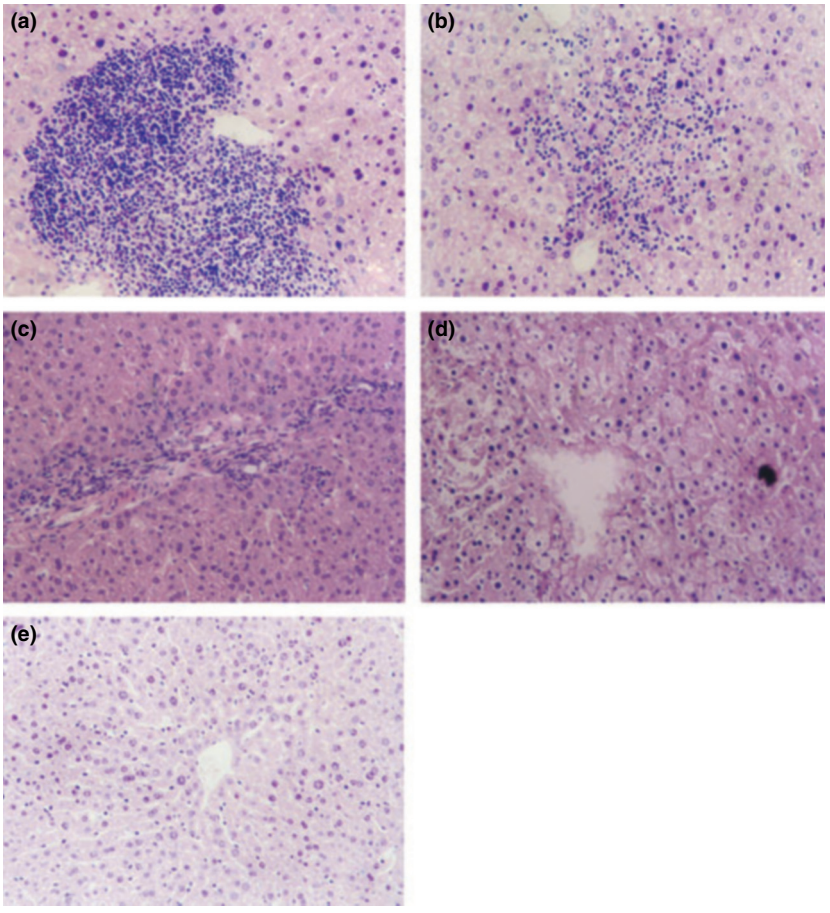

FIG. 2. C57BL/6 mouse liver tissue (haematoxylin and eosin, ×300). (a) Focal hepatocyte necrosis with abundant lymphocyte, macrophage and neutrophil infiltration around the central vein. (b) Focal parenchymal inflammation with moderate lymphocyte and neutrophil infiltration and hepatocellular necrosis. (c) Mild inflammatory cell infiltration in periportal and perivenous areas. (d) Ballooning degeneration of hepatocytes. (e) Normal liver tissue of uninfected mice.

was also observed in some hepatocytes (Table 2, Fig. 2). The gall bladder mucosa of one *H. pylori*-infected mouse showed thickening of the mucous membrane with mild submucosal lymphocytic infiltration (Fig. 3).

In the control mice inoculated with PBS, only slight increases in lymphoplasmocytic cells were noted in the stroma of three mice, and no significant lesions were observed in gall bladder or liver tissue ($p < 0.05$).

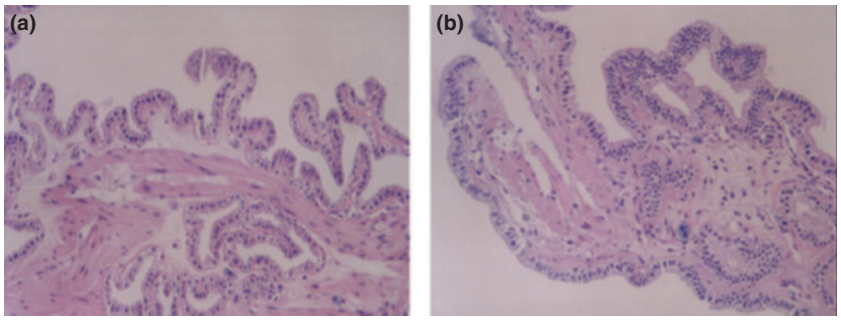

FIG. 3. Gall bladder mucosa of a *Helicobacter pylori*-infected C57BL/6 mouse (×300). (a) Normal gall bladder mucosa. (b) Thickening of mucous membrane with mild submucosal lymphocytic infiltration.

Immunohistochemical staining and carbol fuchsin staining for *H. pylori* in stomach, liver, and gall bladder

Immunohistochemical staining and carbol fuchsin staining for *H. pylori* revealed many helical bacteria in the gastric mucosa of 13 of 15 mice inoculated with *H. pylori*. *H. pylori* was also present in the liver tissues of four mice, often localized between hepatocytes and in lobular central veins (Fig. 4). Spheroid or bacilliform *H. pylori* organisms were also seen in the gall bladder mucosa of six of 15 *H. pylori*-infected mice. No bacteria were found in these tissues in the control group ($p < 0.01$).

PCR amplification for the *H. pylori* 16S rRNA, 26-kDa, *cagA* and *vacA* genes

H. pylori DNA was detected in 14 of 15 gastric samples from mice inoculated with *H. pylori* by nested PCR using specific 16S rRNA primers. The 26-kDa, *cagA* and *vacA* genes were also detected in these 14 samples, which confirmed colonization by *H. pylori* of the gastric mucosa. Taking together the results of *H. pylori* culturing, staining and PCR, the colonization rate was 93.3%. The four above-mentioned genes were also detected in six liver samples of infected mice. Six samples of bile and two blood samples from mice inoculated with *H. pylori* were also positive for the 16S rRNA, 26-kDa, *cagA* and *vacA* genes (Table 2). No *Helicobacter* genes were detected in the control group ($p < 0.01$).

Discussion

It has been reported that some *Helicobacter* spp. (e.g. *H. pylori*, *H. pullorum*, *H. bilis*, and *Helicobacter* sp. flexispira) may be present in intrahepatic or extrahepatic bile ducts in humans. *Helicobacter* DNA (including *H. pylori* DNA) has also been reported in the liver of patients with HCC in several studies from various geographical locations [6–8,14], and this raises the possibility that *Helicobacter* infection may contribute to the pathogenesis of HCC. In our previous study, we also found that *Helicobacter* DNA was present in samples from liver with HCC. DNA sequencing and specific gene detection showed that the *Helicobacter* sp. was *H. pylori* [10,11]. These reports have suggested that *Helicobacter* organisms, including *H. pylori*, may colonize the liver and possibly play a pathogenic role in the development of hepatobiliary disease in humans.

The presence of *H. pylori* in the liver tissues of patients may be explained in two possible ways. It may be a consequence of hepatobiliary disease or, alternatively, *H. pylori* may be involved in the genesis of hepatobiliary disease in humans. In the present investigation, *H. pylori* DNA was detected in six liver samples from mice inoculated with *H. pylori* orally. Carbol fuchsin staining and immunohistochemical staining also revealed morphological evidence of *H. pylori* in four of six liver tissue samples positive for *H. pylori* DNA. Three mice inoculated with *H. pylori* showed slight to moderate mutifocal hepatitis in parenchymal, perivenular and periportal areas, with predominantly lymphocytic infiltration, and these changes are analogous to *H. pylori*-induced lymphoepithelial lesions in chronic gastritis or gastric mucosa-associated lymphoid tissue lymphoma [15]. These results suggest that *H. pylori* can not only spread to the liver in C57BL/6 mice colonized with *H. pylori* by oral inoculation, but also cause pathological changes in the liver. Unfortunately, *H. pylori* was

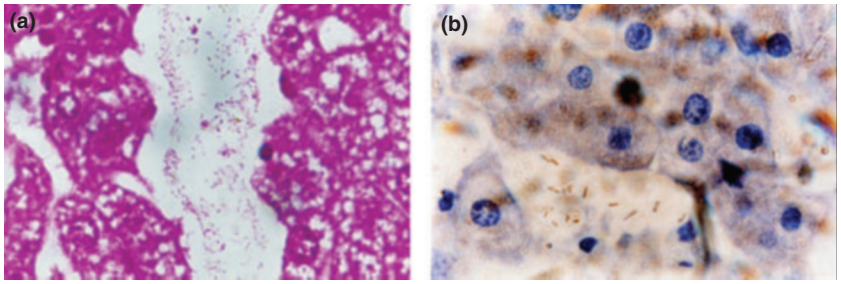

FIG. 4. *Helicobacter pylori* staining in liver tissue of a C57BL/6 mouse (×600). (a) Carbol fuchsin staining. (b) Immunohistopathological staining.

not isolated by culture from the livers of mice in this study, just as it could not be isolated from the liver samples of patients with HCC. The reasons are not clear, as we have discussed in our previous study [10]. Increasing the number of sites in the liver that are sampled may increase the positivity rate for *H. pylori* culture. The organism, however, was more consistently shown to be present in the livers of the *H. pylori*-infected mice by PCR analysis and immunohistochemical staining.

It is not known how *H. pylori* causes inflammation or necrosis in the liver. The importance of bipolar flagella and urease in colonization of gastric mucus by *H. pylori* has been demonstrated *in vivo* by studies using isogenic mutants lacking flagella or this enzyme [16,17]. It will be interesting to ascertain whether these phenotypic features of motility and urease activity in *H. pylori* are also required for the organism to colonize and persist in livers of mice. Urease produces ammonia as a byproduct of urea metabolism, and large amounts of ammonia could cause tissue damage, activate neutrophils, and generate inflammatory cytokines [18,19]. In addition, *H. pylori* produces a hepatocytotoxin *in vitro*, and this protein may play a role in tissue damage *in vivo* [14,20].

Studies have shown that mice inoculated intraperitoneally with *H. pylori* develop acute hepatitis [21]. We established the mouse model of *H. pylori* infection by inoculating orally instead of intraperitoneally, as the usual route of *H. pylori* infection is faecal–oral. Our study showed that the mice inoculated orally with *H. pylori* also developed hepatitis. We may ask by which route *H. pylori* can spread to the liver. In our previous study, we found *H. pylori* DNA in the peripheral blood of patients with duodenal ulcers infected with *H. pylori*. In the present study, *H. pylori* DNA was detected in two blood samples [22], which suggested that *H. pylori* may spread to the liver by the blood-borne route. Several factors are known to lead to increased translocation of microbes across the gastrointestinal barrier. These include disruption of the ecology of the indigenous gastrointestinal microflora leading to bacterial overgrowth, impaired host immunity, and physical disruption of the intestinal mucosal barrier by direct chemical injury, endotoxin, or haemorrhagic shock [23]. *H. pylori* infection can decrease gastric mucin synthesis, produce a striking inflammatory response, affect microcirculatory variables such as blood flow and leukocyte activity, and induce changes in the endothelial lining of the vessels themselves. These effects may impair the gastric mucosal barrier and contribute to mucosal injury, leading to *H. pylori* invading the bloodstream [24,25]. *H. pylori* was not cultured from blood in this study. It may be that *H. pylori* was present at levels below that required for successful culture. Some research groups have reported that *Helicobacter* spp., including *H. pylori*, can be identified in bile or gall bladder tissue in patients with hepatobiliary diseases [26–28]. We also detected *H. pylori* DNA in six bile samples from mice inoculated with *H. pylori*. We postulate that it is possible for *H. pylori* to invade the liver and gall bladder via the bile duct.

In our study, no pathological changes of HCC were found in liver tissue. Wang *et al.* [29] reported that one C57BL/6 mouse infected with *H. pylori* strain 119p developed HCC after 23 months. Further longitudinal studies are warranted to determine whether *H. pylori* infections are involved in the development of HCC.

In conclusion, this study showed that *H. pylori* inoculated orally mainly inhabited the gastric mucosa and caused inflammation or ulceration at this site, but some *H. pylori* organisms could reach the hepatobiliary system and cause inflammation as an independent aetiological factor. Although only three mice out of 15 showed mild to moderate mutifocal hepatitis, the results suggested that *H. pylori* may play a role in such conditions. Longitudinal studies in a larger number of mice should be performed to confirm the results.

Transparency Declaration

There is no dual or conflicting interest among each author.

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